region, operatively linked so that the promoter enhances transcription of the nucleic acid molecule in a host cell. The invention also includes a system for the expression of GRF4, comprising an expression vector and a nucleic acid molecule of the invention molecule inserted in the expression vector. The invention also includes a cell transformed by the expression vector of the invention. Another aspect of the invention relates to a method for expressing polypeptide by transforming an expression host with an expression vector including and culturing the expression host.

The invention also includes a pharmaceutical composition, including all or part of the polypeptide or mimetic of the invention, and a pharmaceutically acceptable carrier, auxiliary or excipient. Another aspect of the invention relates to a GRF4 specific antibody targeted to a region selected from the group consisting of the C-terminus, the CDC25 domain and the PDZ domain.

The invention includes a method of medical treatment of a disease, disorder or abnormal physical state, characterized by excessive GRF4 expression, concentration or activity, comprising administering a product that reduces or inhibits GRF4 polypeptide expression, concentration or activity. The invention also includes a method of medical treatment of a disease, disorder or abnormal physical state, characterized by inadequate GRF4 expression, concentration or activity, comprising administering a product that increases GRF4 polypeptide expression, concentration or activity.

BRIEF DESCRIPTION OF THE DRAWINGS

Preferred embodiments of the invention will be described in relation to the drawings in which:

Figure 1. Domain organization of Rat Nedd4.

Figure 2. Protein sequence of Clone 7.7, the homolog of human clone KIAA0313.

Figure 3A. Schematic Diagram of GRF4.

Figure 3B. Shows the nucleic acid molecule that is [SEQ ID NO:1] and the polypeptide that is [SEQ ID NO:2]. In a preferred embodiment, the figure shows GRF4.

Figure 4. Protein sequence alignment of CDC25 domains from several RasGEF/GRF including GRF4. The CDC25 domain of human GRF4 (hGRF4) was

aligned with those of *Drosophila* GRF4 (dGRF4 [SEQ ID NO:7], identified from genomic DNA sequence [Accession number. AC005285, nucleotide sequence 122129-174319]), human Epac (hEpac), mouse RasGRF2 (mRasGRF2) [SEQ ID NO:9], *Drosophila* SOS (dSOS) SEQ ID NO:10 and RasGRP (hRasGRP) [SEQ ID NO:11]. The three structurally conserved regions present in CDC25 domains are lighter Both hGRF4 and dGRF4 contain a unique insertion shown in blue. Alignments were created using the program Clustal W(1.7).

Accession numbers.
hGRF4 (AB002311), dGRF4(AC005285), hEpac(AF103905), mRasGRF2(U67326), dSOS(M83931), hRasGRP(AF106071), rLin-7-C(AF090136), hPTP-BAS-1(D21209), hDlg(U61843), hPRKAR1B(M65066), hPSD-95 (AF156495), hPKGII(CAA76073), mEAG(U04294).

Figure 5. Protein sequence of alignment of Ras GRF4-REM domainincluding CDC25 [SEQ ID NO:12], Sos_mouse_[SEQ ID NO:13], GRF2_mouse_[SEQ ID NO:14]. RasGEF_aimless_[SEQ ID NO:15].

Figure 6A. Overall structure comparison between GRF4 and other known mammalian GRFs/GEFs which activate Ras.

Figure 6B. An example of the most well known Ras signaling pathway.

Figure 7. Sequence alignment of GRF4-PDZ domain. The PDZ domains of hGRF4 and dGRF4 [SEQ ID NO:16] were aligned with those of rat Lin-7-C (rLin-7-C) [SEQ ID NO:19], human PTP-BAS type 1 (hPTP-BAS-1) [SEQ ID NO:17], human Dlg (hDlg) [SEQ ID NO:20] and human PSD-95 (hPSD-95) [SEQ ID NO:18]. The sequences corresponding the GLGF motif present in prototypic PDZ domains are lighter.GRF4 Alignments were created using the program Clustal W(1.7).

Figure 8. Sequence alignment of GRF4-cNMP-BD. The cNMP-BD of hGRF4 was aligned with those of dGRF4 [SEQ ID NO:21], hEpac [SEQ ID NO:22], human cAMP-dependent protein kinase regulatory subunit type 1b (hPRKAR1B) [SEQ ID NO:23], human cGMP dependent protein kinase (hPKGII) [SEQ ID NO:24], and mouse cyclic nucleotide gated potassium channel (mEAG) [SEQ ID NO:25]. The conserved motifs RAA present in hPRKAR1B and hEpac that confers cAMP binding specificity are shaded in blue. The conserved motifs RTA present in hPKGII and mEAG that confers cGMP binding specificity are lighter. Alignments were created using the program Clustal W(1.7).

Figure 9. Protein sequence alignment of GRF4-RA domain including dgk-1a_ce_[SEQ ID NO:26 and RalGDS_h_[SEQ ID NO:27].

Figure 10. Tissue Distribution of GRF4.

Figure 11. Co-precipitation of endogenous Nedd4 in Hek 293T cells by a GST-fusion protein of the C-terminal last 150 aa of GRF4 which contains the two PY motifs .

Figure 12. Co-immunoprecipitation of GRF4 with endogenous Nedd4 in Hek 293T cells transiently transfected with Flag-tagged GRF4.

Figure 13. Method used for the *in vitro* GEF assay.

Figure 14. *In vitro* GEF assay using immunoprecipitated full-length GRF4 demonstrating activation of Ras by GRF4 (additional data in Fig. 23(e)).

Figure 15. GRF4 forms stable complex with GST-Ras in vitro.

Figure 16. GRF4 induces foci formation in Rat2 fibroblasts.

Figure 17. GST-fusion protein of GRF4-PDZ domain binds full-length GRF4 expressed in Hek 293T cells.

Figure 18. Biotinylated peptide of the last 15 amino acid sequence of GRF4 containing a PDZ-binding motif (SAV*) binds full-length GRF4.

Figure 19. (a) Nucleic acid molecule sequence [SEQ ID NO:1] and amino acid sequence [SEQ ID NO:2]; (b) The figure shows the nucleic acid molecule sequence that is [SEQ ID NO:3] and amino acid sequences [SEQ ID NOS:4,5,6]. In a preferred embodiment, [SEQ ID NO:3] is the Clone 7.7 DNA nucleic acid molecule sequence

Figure 20. Plasma membrane localization of GRF4.

Figure 21. GRF4 domain organization and expression. (a) GRF4, depicting its cNMP (cAMP/cGMP) binding domain (cNMP-BD), a Ras Exchange Motif (REM), a PDZ domain, a Ras Association (RA) domain, a CDC25 domain which contains an insert region (white box) and a C terminus which includes 2 PY motifs (PPxY) that bind Nedd4 WW domain(s). The COOH terminus ends with the sequence SAV, conforming to a PDZ binding motif. Sequence alignment of the CDC25, cNMP-BD and PDZ domains is provided in the Supplementary material.

(b) Northern blot analysis of GRF4 mRNA in multiple regions of human brain, probed with the radiolabelled cDNA corresponding to the 3' region of human GRF4 (nucleotides 4286-4620 of KIAA0313), and depicting expression of ~7.5 and ~8.5 kb

size transcripts. (blot purchased from Clontech). A multiple rat tissue Northern blot (from Clontech) probed with GRF4 cDNA revealed strong expression primarily in the brain (not shown).

(c) Western blots depicting characterization of anti GRF4 antibodies and expression of the GRF4 protein in synaptosomes. Polyclonal anti GRF4 antibodies were raised against a GST-fusion protein encompassing the C terminus (amino acids 1350-1499) of GRF4, and recognize the ~180 kDa GRF4 protein either heterologously expressed in HEK-293T cells (epitope-tagged with HA, Flag (Fl) or myc tags) (left panel), or endogenously expressed in synaptosomes from adult (Ad) or embryonic (Emb) rat brain (right panel). No protein was detected with the pre-immune (pre-imm) serum. tfxn, transfection; IP, immunoprecipitation; αGRF4, anti GRF4 antibodies.

Figure 22. Binding of cAMP to the cNMP- binding domain (cNMP-BD) of GRF4.

(a) *In vitro* binding of GST-GRF4-cNMP-BD, but not GST alone, to immobilized cAMP. cAMP-agarose beads were incubated with soluble GST-GRF4-cNMP-BD or GST alone, washed, proteins separated on 10% SDS-PAGE and immunoblotted with anti GST antibodies (upper panel). Total amount of proteins incubated with the cAMP beads is shown in the lower panel (coomassie).

(b) Precipitation of transfected GRF4, but not mutant GRF4 lacking its cNMP-BD (_cNMP-BD), with cAMP agarose beads. cAMP agarose beads were incubated with cell lysates from HEK-293T cells expressing either GRF4 or _cNMP-BD, followed by washing of beads, SDS-PAGE, and immunoblotting with anti GRF4 antibodies (upper panels). Expression of full length and mutant GRF4 was verified by immunoblotting aliquots of the respective cell lysates with the same antibodies (bottom panels). Right and left panels in (b) represent two separate experiments.

Figure 23 cAMP/cGMP-mediated activation of Ras, but not Rap1, by GRF4 in living cells. (a) cAMP-dependent and PKA-independent activation of ras by GRF4.

HEK-293T cells were transfected (or not) with Flag-tagged GRF4, serum-staved overnight, pre-treated (or not) with the PKA inhibitors H-89 (10 _ M) or Rp-cAMPS (50 _ M) for 30 min., and then treated (or not) with the cAMP analogue 8-Br-cAMP (500 _ M) for 15 min. Cells were then lysed and lysate incubated with immobilized Ras binding domain (RBD) of Raf1 (GST-Raf1-RBD), which binds activated (GTP-bound) Ras. Co-precipitated activated ras was then detected with anti Ras antibodies (Quality Biotech) (upper panel). Lower 2 panels depict the amounts of total